U. S. Department of Commerce
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Secretary
National Bureau of Standards
Richard W. Roberts, Director

# National Bureau of Standards Certificate of Analysis

## Standard Reference Material 921

### Cortisol (Hydrocortisone)

B. Coxon and R. Schaffer

This Standard Reference Material is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of procedures for cortisol determinations employed in clinical analysis and for routine critical evaluation of the daily working standards used in these procedures.

| Constituent | Percent |
|---|---|
| Cortisol | 98.9 |
| 21-Dehydrocortisol | 0.6 |
| 21-O-Acetylcortisol | 0.2 |
| 21-Dehydrocortisone | 0.1 |
| Cortisone | 0.1 |
| Total Steroids | 99.9 |
| Ash | 0.002 |
| Insoluble matter | 0.001 |
| Loss on drying | 0.08 |

The cortisol assay has an estimated inaccuracy of 0.2 percent.

The cortisol used for this Standard Reference Material was obtained from the Upjohn Company, Kalamazoo, Michigan. Analyses were performed by R. F. Brady, Jr., A. Cohen, B. Coxon, M. Darr, W. D. Dorko, D. P. Enagonio, T. E. Gills, E. E. Hughes, W. P. Schmidt, and S. A. Wicks of the Analytical Chemistry Division.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

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J. Paul Cali, Chief
Office of Standard Reference Materials

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Identification and quantitation of the four steroid impurities were accomplished by Fourier-transform proton-magnetic-resonance (pmr) spectroscopy and thin-layer chromatography (tlc) performed on fractions from the liquid chromatography of 100 mg of the material in ethanol on a column (91 x 0.8 cm) of poly(vinylpyridine) crosslinked with 8 percent of divinylbenzene. Fractions were eluted with ethanol at a pressure of 4.7 $kg \cdot cm^{-2}$. Tlc of aliquots of the fractions was performed on silica gel $GF_{254}$ using 9:1 (v/v) chloroform-methanol. Equivalent sensitivity of detection was obtained by fluorescence quenching and by spraying with aqueous 20-percent sulfuric acid and heating at 120 °C. All the steroid impurities, except 21-dehydrocortisone, showed similar sensitivity. 4-Androsten-11β-ol-3,17-dione was found in liquid chromatography fractions, but could not be demonstrated directly by tlc of up to 1 mg of the bulk material. Because this compound was readily resolved from mixtures prepared with it and the bulk material and detected in proportion to amounts used for reference, the compound was adjudged an artifact produced during liquid chromatography. On the other hand, the major impurity, 21-dehydrocortisol, did not arise by the known copper-catalyzed oxidation of cortisol, as shown by its unaltered proportion by tlc of the bulk material even after EDTA-treatment of the system to remove copper.

The quantitative proportion of each steroid present in the sample was estimated from the dry weight (W) of the residue of each liquid chromatographic fraction and the measured intensity of the Fourier-transform generated methyl signals characteristic for each steroid, using the expression:

$$m_n M_n = \frac{W h_n M_n}{h_1 M_1 + h_2 M_2 \ldots + h_m M_m}$$

where $m_n$ and $M_n$ are the number of moles present and the molecular weight, respectively of the $n_{th}$ component in a mixture of m components, $h_n$ is the methyl signal intensity of the $n_{th}$ component, and $h_1 \ldots h_m$ are the corresponding intensities of components 1...m, obtained by measurement of the methyl peaks above the methylene envelope of the steroids.

For proof of homogeneity, nine samples were withdrawn from the bulk Standard Reference Material according to a statistical plan. They were analyzed in a commercial carbon-hydrogen-nitrogen microanalyzer and were found to be homogeneous with respect to carbon and hydrogen content within the limits of precision of the method. Solutions of the samples in 95 percent ethanol at 25 °C showed an absorbance maximum at 242 nm with a molar extinction coefficient of 16.1 x $10^3$ $liter \cdot cm^{-1} \cdot mol^{-1}$.

Elemental macroanalysis of the material showed carbon 69.49 ± 0.10 percent (2 SD of the mean); and hydrogen 8.39 ± 0.05 percent (2 SD of the mean); the calculated values for cortisol are 69.58 percent and 8.34 percent, respectively.

The Standard Reference Material melted at 219.0-220.5 °C (corrected) when heated in an open capillary tube at 0.5 $°C \cdot min^{-1}$. The resulting pale yellow melt did not solidify on cooling. After sealing in a capillary tube under vacuum, the material melted at 220.5-221.5 °C without yellowing, but did not resolidify.

Thermogravimetric analysis of samples heated under dry nitrogen at 2 $°C \cdot min^{-1}$ showed the initiation of loss of a large proportion of sample weight at 221 °C (uncorrected). However, for samples heated in air, loss of weight began at 204 °C. The attempted application of differential scanning calorimetry to samples heated under nitrogen gave thermograms that were very dependent on the rate and time of heating, and that were not reproducible.

The mass spectrum of the Standard Reference Material obtained by electron induced ionization at 70eV and a probe temperature of 220 °C showed strong ion currents at *m/e* 362 [$M^{+\cdot}$], 344 [M−18($H_2O$)], 332 [M−30($CH_2O$)], 303 [M−59($COCH_2OH$)], 302 [M−60($CH_2$=C=O and $H_2O$)], 285 [M−59−18], 42 [$CH_2$=C=O], and 31 [$CH_2$=O−H]$^+$. The peaks at *m/e* 302 and 163 correspond to the molecular ion and key fragment, respectively, of the thermal degradation product of cortisol, namely 4-Androsten-11$\beta$-ol-3,17-dione.

Optical rotations of solutions of the Standard Reference Material in ethanol (c 1.00) were measured at 20 °C by means of automatic and high-precision, manual polarimeters and are as follows:

| | $[\alpha]_\lambda^{20}$ (±2 SD of the mean) | |
|---|---|---|
| λ (nm) | degrees | radians |
| 589 | 168.9±0.1 | 2.948±0.002 |
| 578 | 177.3±0.1 | 3.094±0.002 |
| 546 | 205.6±0.1 | 3.588±0.002 |
| 436 | 387.9±0.4 | 6.770±0.007 |
| 365 | 571.7±0.6 | 9.978±0.010 |

Insoluble matter was determined by filtration of solutions of the Standard Reference Material (1.8-2.0 g) in methyl sulfoxide (9-20 ml) through Millipore filters (1.5 $\mu$m).

The loss of weight on drying was determined after heating three samples (2.8 g, each) of the material at 110 °C and 0.1 torr for 24 hours. No further weight loss occurred during heating of the samples for an additional 48 hours.

Ash was determined by heating of samples (10 g) of the Standard Reference Material to 700 °C. Spectrometric analysis of the ash indicated calcium as a major constituent, and sodium, magnesium, and silicon as minor constituents.

Destructive neutron activation analysis using SRM 1577, Bovine Liver, as a standard indicated that the Cortisol Standard Reference Material contains 0.035±0.005 ppm (±2 SD of the mean) of copper.

This Standard Reference Material is for "in vitro" diagnostic use only.

A standard solution containing 5 $\mu$g per ml of cortisol may be prepared as follows. Transfer 50 mg of SRM 921 to a 50-ml volumetric flask and add 35 ml of absolute ethanol. When the cortisol is completely dissolved, dilute to the mark with absolute ethanol. Dilute 1 ml of this ethanol solution to 200 ml with distilled water [1,2].

Working standard solution may be made by appropriate dilution of aliquots of the 5 $\mu$g per ml solution with distilled water.

This Standard Reference Material should be stored in a well-closed container at room temperature (30 °C or less). It should not be subjected to heat or direct sunlight during storage. Refrigerated storage is recommended. Under proper storage, experience at NBS indicates purified cortisol to be stable for at least 5 years. If the material purity degrades beyond the limits certified, purchasers will be notified by NBS. It is recommended that material not be used after 5 years from date of purchase.

The solution of 1 mg/ml of cortisol in ethanol as prepared above should be stored in a well-stoppered, all-glass container and kept in a refrigerator at 4 °C. The aqueous solution of 5$\mu$g per ml cortisol should also be stored in a well-stoppered, all-glass container at 4°C. Under these conditions both solutions should be stable for six months. The more dilute "working" standard solutions should be prepared daily.

All constituted solutions of cortisol should be clear and display no turbidity.

References:

[1] R. E. Peterson, "Hydrocortisone in plasma", in Standard Methods of Clinical Chemistry, Vol. 3, David Seligson, Editor-in-Chief, pp. 160-166, Academic Press, Inc., New York, N. Y. (1961).

[2] N. W. Tietz, Fundamentals of Clinical Chemistry, pp. 509-512. W. B. Saunders Company, Philadelphia, Pa., (1970).

This Standard Reference Material has been measured and certified at the laboratories of the National Bureau of Standards, Gaithersburg, Maryland. All inquiries should be addressed to:

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